IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Curiel & Tillman	§	**ART UNIT: 1632**
	§	
FILED: June 12, 2000	§	**EXAMINER:**
	§	**Li, Q.**
SERIAL NO.: 09/591,737	§	
	§	
FOR: Immunomodulation By Genetic Modification of Dendritic Cells And B Cells	§	**DOCKET: D6167CIP**

The Assistant Commissioner of Patents
BOX NON-FEE AMENDMENT
Washington, DC 20231

DECLARATION UNDER 37 C.F.R. § 1.132

Dear Sir:

I, David T. Curiel, does hereby state as follows:

I am a co-inventor of the above-referenced patent application. I have read U.S. patent application serial no. 09/591,737 and I am aware of the content of the Office Action, including all prior art cited against the '737 application.

The Examiner contends that there is lack of sufficient enablement for using the CD40-targeted adenoviral vectors of the instant invention in human. Claims 11-16, 27-28 are drawn to methods of using the gene delivery system of the instant invention to genetically modifiy $CD40^+$ immune cells in an individual. The gene delivery system of the instant invention can both mediate gene transfer to and cause maturation of $CD40^+$ immune cells. Claims 17, 19-21, 23, 24, 29-30, 40-45 and 53-56 are drawn to methods of using the CD40-targeted adenoviral vectors of the instant invention to enhance vaccination potential of dendritic cells. The following data are presented as evidence of enablement

CD40-Targeted Ad Transfer To Human Ovarian Cancer Cells

CD40 was found to be expressed on various human ovarian cancer cells. Subsequently, the potential of this phenomenon for targeting adenovirus was examined. The ovarian cancer cells were infected with three doses of non-targeted (Ad5luc1) or CD40-targeted (Ad5luc1 + CAR/G28) adenoviruses (Fig. 1). With the CD40 negative HeLa cells, CAR/G28 conferred no advantage in gene expression (Fig. 1F). In contrast, all CD40 positive cell lines (Fig. 1A-E) demonstrated notable enhancement in gene transfer efficacy when CD40-targeted virus was used. When 100 pfu/cell of Ad5Luc1/CAR/G28 was used, gene transfer was increased 4, 6, 42, 13 and 8 fold for Hey, SKOV3.ip1, OV-4, OV-3 and BT-20 cells respectively in comparison to Ad5Luc1 alone (Fig. 1).

Transduction Of Human Skin DC: Increased Selectivity After CD40-Targeted Ad Transfer

Human skin explants were injected i.d. with 100 ng GM-CSF in combination with an unconjugated adenoviral vector encoding *LacZ* (Ad-*LacZ*) or with Ad-*LacZ* complexed to a chemically linked bispecific antibody conjugate directed to the fiber knob region of the Ad capsid and to CD40 (Fab-anti-CD40). The explants were harvested and snap-frozen 48 hours after i.d. injection, allowing for an additional 24h after optimal CD40 expression levels had been reached in order to ensure expression of the gene of interest. β-Galactosidase (β-Gal)-expressing cells were visualized and scored on cryosections. The optimal dose of Ad-*LacZ* was determined to be 10^8 pfu per injected

number of cells within the dermis, but not in the epidermis. Similarly, the injection of Fab-anti-CD40-complexed Ad-*LacZ* only led to the transduction of cells in the dermis. However, CD40 targeting of Ad resulted in a drastic reduction in the number of transduced cells, indicating an effective block of the natural tropism of the Ad vector.

Numbers of β–Gal positive cells were counted by microscopic examination in ten randomly chosen high power fields. Double staining with a PE-labeled antibody demonstrated almost all (typically more than 99%) of the untargeted adenovirus-transduced cells to be CD1a negative. In contrast, injection of Fab-anti-CD40-complexed Ad-*LacZ* resulted in a substantial increase in the proportion of CD1a+ transduced cells (at a mean of 44%), thus revealing a more selective targeting of the adenoviral vector to dendritic cells. In parallel, Ad-*LacZ* complexed to Fab-anti-EGFR (a bispecific antibody conjugate targeting Ad to the epidermal growth factor receptor) was also injected into skin explants. No β-gal activity was detected using this conjugate, indicating effective blocking of the natural tropism of adenovirus and excluding the possibility of adenovirus uptake by binding of antibodies in the conjugate to Fc receptors on the surface of dendritic cells. These data indicate that the increased selectivity of cutaneous dendritic cells transduction is due to specific binding to CD40.

CD40-Targeted Ad Infection In Relation To Maturation State And Migration Of Human Cutaneous dendritic cells

The effect of cytokine-induced maturation on the transduction efficiency of migrated cutaneous dendritic cells by untargeted or CD40-targeted adenovirus was

CSF with 1000 U IL-4 prior to the 48h culture of the skin explants were examined. Under all these conditions CMRF-44 expression was found on the migrated dendritic cells, indicative of early maturation. Further maturation was induced after the i.d. injection of GM-CSF and/or IL-4, evidenced by upregulation of CD83 and CD40 expression (Figure 2a). The most mature phenotype was consistently achieved through the simultaneous injection of GM-CSF and IL-4 (Figure 2a). Accordingly, highest numbers of migrating dendritic cells were always found after i.d. injection of GM-CSF in combination with IL-4. The ability of untargeted or CD40-targeted Ad to infect cutaneous DC *in situ* in a mature state after GM-CSF and IL-4 injection was subsequently compared to the more immature control condition after injection of plain medium.

Skin explants were i.d. injected with either plain medium or medium containing GM-CSF and IL-4, and cultured on nitrocellulose filter rafts at the medium/air interface for 24 h to allow for dendritic cell maturation prior to i.d. injection of 10^8 pfu of untargeted or CD40-targeted Ad-vectors encoding the Green Fluorescent Protein (GFP). The explants were subsequently placed directly into culture medium and cultured for another 24h, after which migrated DC were harvested and analyzed by FACS. Significant and comparable increases in transduction efficiency of migrated dendritic cells by CD40 targeting of Ad were observed in both test conditions (Figure 2b). Following medium injection, Ad administration led to a mean transduction efficiency of 35.3% (range 14.9-47.7%), while CD40 targeting increased the mean transduction efficiency to 59.0% (range 30.0-85.6%) (n=5, P=0.005). Corresponding transduction efficiencies after GM-CSF and IL-4 injection were 44.6% (range 16.1-58.0%) and 73.4% (range 59.0-84.0%),

respectively (n=7, P=0.0003). Similarly, expression levels of the transduced GFP gene were significantly increased through CD40 targeting (Figure 2b).

FACS analyses of migrated cutaneous dendritic cells revealed upregulation of maturation markers after CD40-targeting of Ad *in situ*. Although dendritic cells migrating from medium- and GM-CSF/IL-4-injected skin explants already displayed a (partially) mature phenotype on the basis of CD83 expression (Figure 2c), this could be further upregulated in both cases by the injection of CD40-targeted, but not of untargeted Ad vectors (Figure 2c). A simultaneous upregulation of CD80, CD86, CD54, and HLA-ABC accompanying CD40-targeting was also observed (Figure 2c). CD40 targeting of Ad consistently led to the migration of significantly higher numbers of transduced, GFP-expressing dendritic cells, with the highest absolute number of transduced DC migrating from the explants pre-injected with GM-CSF and IL-4.

Long Term Transgene Expression And Maturation Of Human dendritic cells After CD40 Targeting

The long term effects of CD40-targeting of adenovirus on transgene expression and dendritic cell activation state were examined in dendritic cells migrated from explants, pre-injected with plain medium without cytokines. Migrated cutaneous dendritic cells were harvested as before, but cultured in cytokine-free medium for an additional five day-period. Transduction efficiencies and transgene expression levels for the different targeting conditions were comparable to those found immediately after migration, with similarly increased levels of transduction after CD40 targeting (Figure 3a).

conditions. However, CD83 expression, indicative of persistent activation, by this time was only found on dendritic cells transduced by CD40-targeted Ad (Figure 3a). Moreover, this persistence of maturation corresponded with the dendritic cells' ability to stimulate allogeneic T cells in the mixed lymphocyte reaction culture (Figure 3b).

In summary, these data demonstrated a more selective and enhanced transduction of cutaneous dendritic cells through the targeting of adenovirus to CD40 *in situ*. This transduction was accompanied by an increased and prolonged activation of dendritic cells subsequent to migration. Dendritic cells infected by untargeted adenovirus were found to acquire a T cell non-stimulatory phenotype over time, which *in vivo* might ultimately lead to a transgene-specific T cell unresponsiveness. In contrast, CD40 targeting of adenovirus appears to ensure the ability of the transduced dendritic cells to stimulate T cells for at least up to a week after transduction.

The data contained herein show that the gene delivery system of the instant invention can both mediate gene transfer to and cause maturation of $CD40^+$ immune cells. Consequently, I respectfully submit that the scope of the claims 11-16, 27-28 which are drawn to methods of using the gene delivery system of the instant invention to genetically modify $CD40^+$ immune cells in an individual has a reasonable correlation to the scope of the enablement provided.

Claims 17, 19-21, 23, 24, 29-30, 40-45 and 53-56 are drawn to methods of using the CD40-targeted adenoviral vectors of the instant invention to enhance the vaccination potential of dendritic cells. As discussed above, the CD40-targeted adenoviral vectors of the instant invention mediate both gene transfer to and cause maturation of

adenoviral vectors of the instant invention have increased vaccination potential. This is supported by the fact that human dendritic cells infected by untargeted adenovirus were found to acquire a T cell non-stimulatory phenotype over time, whereas data presented herein indicate that CD40 targeting of adenovirus ensure the ability of the transduced dendritic cells to stimulate T cells for at least up to a week after transduction. Moreover, *ex vivo* skin explant cultures were previously used as a model system to study migrational patterns of skin-derived dendritic cells and shown to be representative of active migration of dermal and epidermal (i.e. Langerhans cells) dendritic cells through lymph vessels to the skin-draining lymph nodes. Since vaccine administration through the skin affords excellent tumor protection, data from this human model system presented above demonstrate targeted and enhanced transfer of adenoviral vectors to migrating dendritic cells, as well as their increased maturation, through a CD40-targeting antibody complex. Based on the data presented herein, I respectfully submit that the scope of the claims 17, 19-21, 23, 24, 29-30, 40-45 and 53-56 has a reasonable correlation to the scope of the enablement provided.

Figure Legends

Figure 1: Infectivity of CD40 expressing human ovarian cancer cell lines was enhanced when CD40 retargeted adenovirus was used. Cells were infected with Ad5luc1 (striped column) or with CAR'G28 retargeted Ad5luc1 (black column). Luciferase readings were measured 24h after infection. BT-20 (CD40+) and HeLa (CD40-) were used as controls. The error bars indicate ± 1 SD.

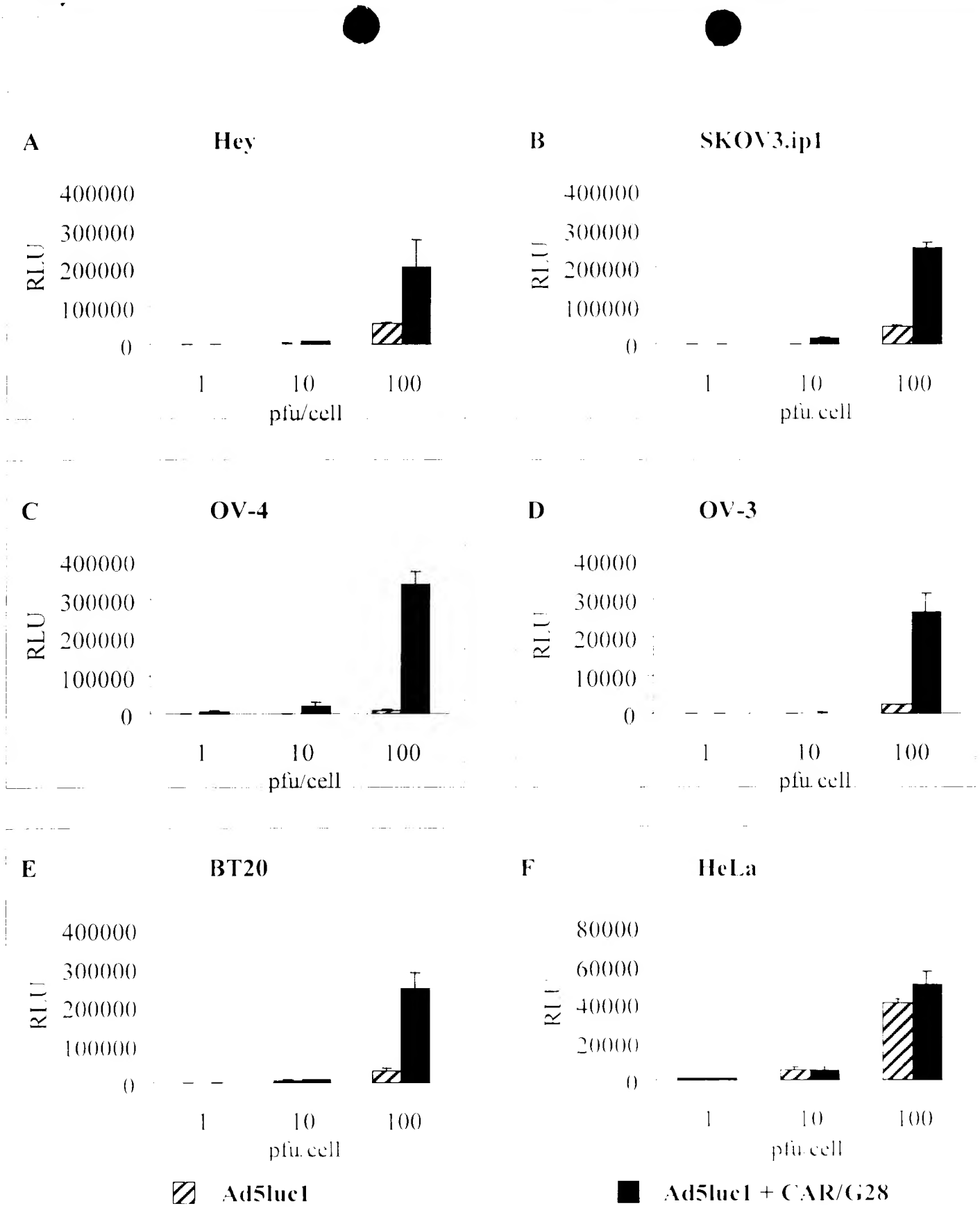
Figure 2: (a) Expression of the DC maturation markers CMRF-44, CD83 and CD40 on dendritic cells migrated from human skin explants 48h after i.d. injection of plain medium, GM-CSF (100ng), or GM-CSF (100 ng) and IL-4 (1000 U). (b) Skin explants were i.d. injected with either plain medium (n=5) or GM-CSF (100 ng)/IL-4 (1000 U) (n=7), and then i.d. injected with 10^8 pfu of untargeted (open bars) or CD40-targeted Ad-GFP (closed bars). 24h later migrated cells were harvested and analyzed for GFP expression by FACS. Transduction efficiencies (in %) and GFP expression levels (in Mean Fluorescence) are shown. Asterisks denote significant differences between the untargeted and CD40-targeted conditions. (c) dendritic cells migrated from skin explants, pre-injected with either medium or GM-CSF/IL-4 and subsequently with untargeted or CD40-targeted Ad-GFP, were labeled with PE-conjugated antibodies to CD83, CD80, CD86, CD54, and HLA-ABC, and analyzed by FACS; mean fluorescence levels for the indicated markers are listed. One representative experiment out of four is shown.

Figure 3: Skin explants were i.d. injected with plain medium, and then injected with medium, 10^8 pfu of untargeted, EGFR-targeted, or CD40-targeted Ad-GFP. 24h later the explants were harvested and cultured for 5 more days in cytokine-free culture medium. (a) The dendritic cells were labeled with PE-conjugated isotype (iso) mAb or CD83 mAb, and analyzed by FACS for GFP and CD83 expression levels. Transduction efficiencies (in % GFP+ cells) are listed. The shown experiment is representative of three. (b) The dendritic cells were tested for their T cell stimulatory ability in an allogeneic mixed lymphocyte reaction (MLR) at the indicated ratios with responder lymphocytes (PBL). Results from one experiment of two are shown.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

DATE:_____ _____

DR. DAVID T. CURIEL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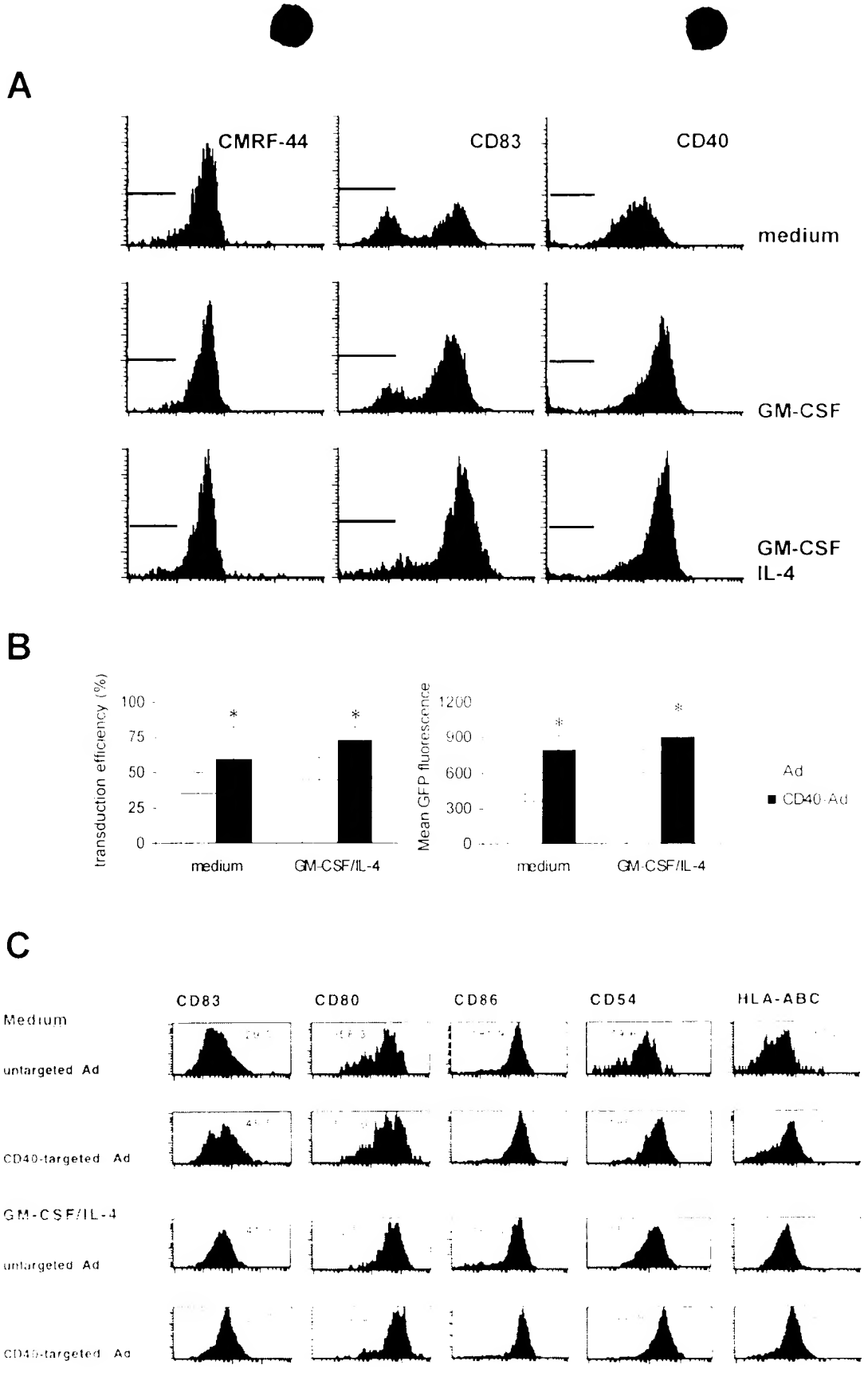
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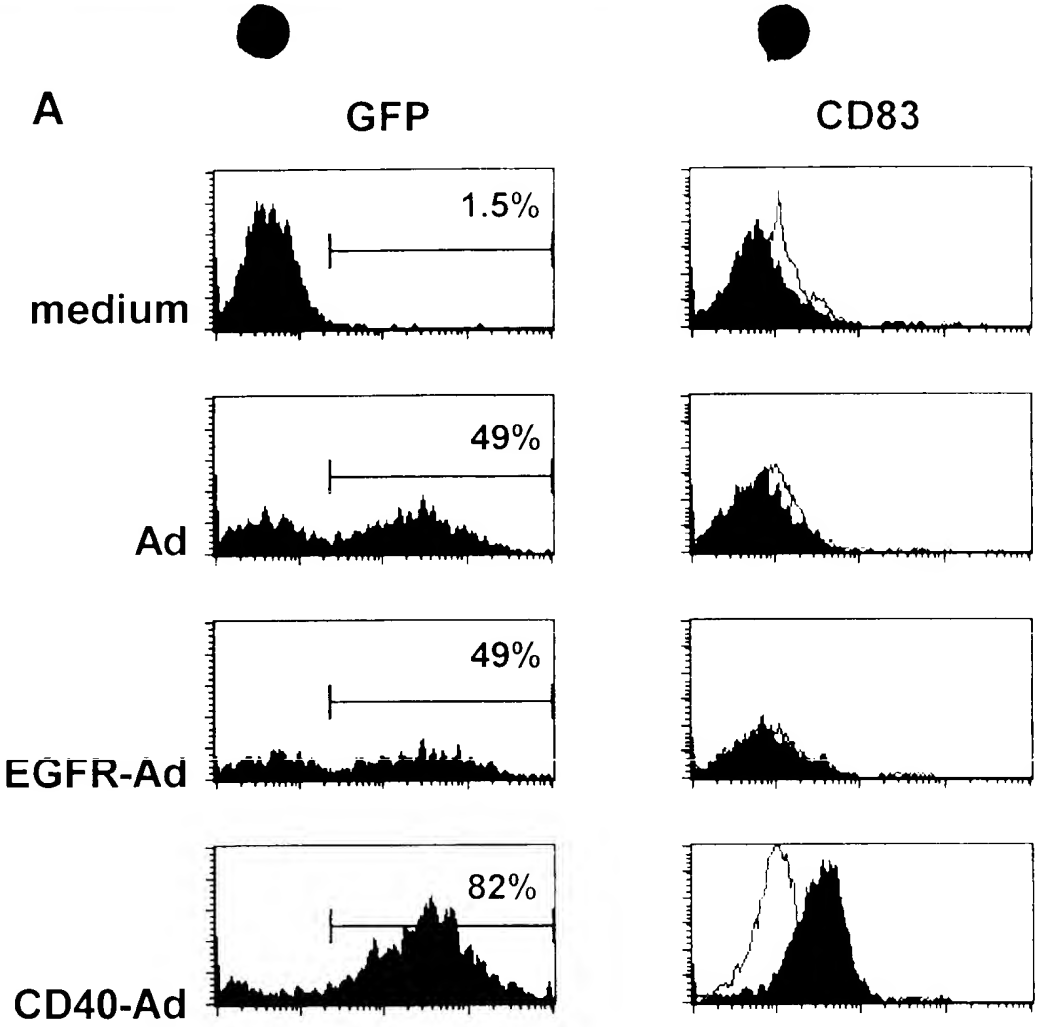
CD40-targeted Ad

Figure 2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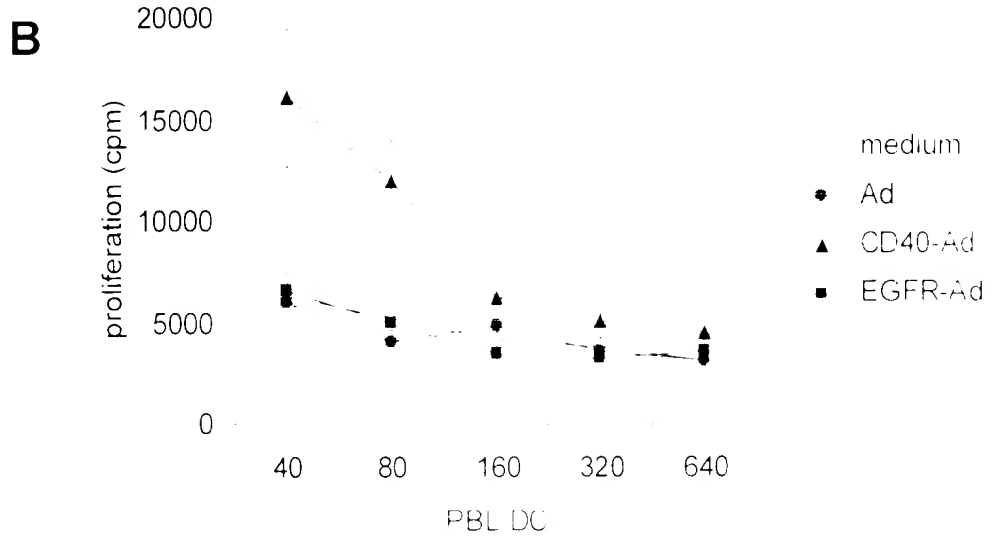
CD40-Ad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